

Synthesis, release and uptake of transmitter amino acids at central nervous synapses

Thesis

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LIST OF PAPERS

Paper I

Holten, A.T.*, Morland C.*, Nordengen, K., & Gundersen, V. Vesicular release of L- and D-aspartate from hippocampal nerve terminals: immunogold evidence. *Submitted*

Paper II

Gundersen, V., Holten, A.T., & Storm-Mathisen, J. GABAergic synapses in hippocampus exocytose aspartate on to NMDA receptors: quantitative immunogold evidence for co-transmission. *Mol. Cell Neurosci.* 26, 156-165 (2004).

Paper III

Holten, A.T. & Gundersen, V. Glutamine as a precursor for transmitter glutamate, aspartate and GABA in the cerebellum: a role for phosphate-activated glutaminase. *J. Neurochem.* 104, 1032-1042 (2008).

Paper IV

Holten, A.T., Danbolt, N.C., Shimamoto K., & Gundersen, V. Low-affinity excitatory amino acid uptake in hippocampal astrocytes: a possible role of Na⁺/dicarboxylate cotransporters. *Glia* 56, 990-997 (2008).

* these authors contributed equally to the work.

INTRODUCTION

The synapse

In the late 19th century there were a lot of agonizing argues between the scientists. One of the most famous, at least within neuroscience, is the argue between the reticularists and the neuronists, personified by the leading neuroscientists and histologists of the time Camillo Golgi and Santiago Ramón y Cajal (Rapport, 2005). The reticularists, with the Italian Golgi in front, believed that the nervous tissue in the brain was made up of one continuous net of branches – a syncytial system. Cajal and the neuronists meant that the neurons in the brain were separated with small gaps (the neuron doctrine). We now understand that they actually argued about the existence of the synapse.

In 1906 Golgi and Cajal shared the Nobel Prize in medicine for their studies of the structure of the nervous system. At the time the neuron doctrine was generally recognized to be the most probable, and even Golgi admitted that in his Nobel lecture (Golgi, 1907).

In 1887, the same year as Cajal started his histological studies, the Norwegian explorer Fridtjof Nansen gave out his doctor thesis about the nervous system of the hagfish. Nansen wrote: “I have not yet observed a single case of indubitable anastomosis between protoplasmatic processes. I believe, thus, that I am entitled to affirm that a direct combination between ganglion cells, by direct anastomosis of the protoplasmic processes, does not exist.” (Nansen, 1887). Nansen ended his neuroscientific career after he was conferred the doctor’s degree. If he had continued, maybe he would have received the Nobel Prize in medicine in 1906. Instead he got another Nobel Prize, namely the Peace Prize in 1922.

Cajal and Nansen were correct about the existence of a gap between the nerve cells. However, they had never seen these gaps, only some small dots in the light microscope representing the nerve terminals. The actual gap was not possible to visualize before the electron microscope was invented.

The gap is a part of the synapse, and this is where the nerve cells communicate with each other (fig 1). Each neuron forms about 1000 synapses with neighboring cells, meaning that the brain consists of about 10^{15} synapses. Understanding synaptic transmission is fundamental in the understanding of the functions of the brain.

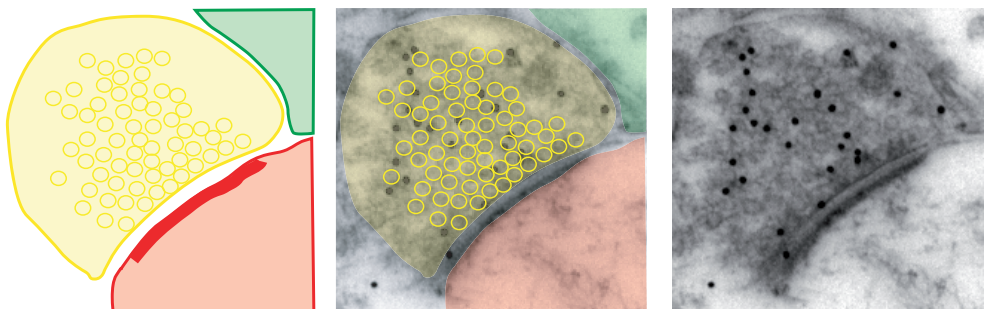


Fig 1: The synapse. The presynaptic terminal (yellow) contacts the postsynaptic dendrite (red). Both are enclosed by glial membranes (green). The cartoon on the left is fused together with the electron micrograph on the right. The black dots on the micrograph are gold particles labeling glutamate.

The chemical synapse consists of a presynaptic and a postsynaptic membrane and a synaptic cleft in between. For a signal to be transmitted from one nerve cell to another, neurotransmitters have to be released from the presynaptic part of the synapse. The neurotransmitters will diffuse across the synaptic cleft and bind to a postsynaptic receptor. This receptor activation is responsible for the effects in the postsynaptic cell, e.g. a depolarization of the cell leading to an action potential.

The presynaptic nerve terminal contains a cluster of vesicles. These vesicles hold the transmitters. The synaptic vesicles located in the “readily releasable pool” just opposite the synaptic membrane, the active zone, will in the presence of increased intracellular Ca^{2+} -concentrations move towards and fuse with the synaptic membrane. The lumen of the vesicles will then be a part of the extracellular space, the synaptic cleft, and the transmitters will diffuse freely. This process is called regulated exocytosis.

The postsynaptic receptors are located on the other side of the synaptic cleft. Each of the different transmitters has a specific set of receptors. The receptors are membrane-spanning proteins with specific binding sites for transmitters.

Action potentials in nerve terminals lead to release of transmitters

In most nerve cells the resting membrane potential is about -65 mV. This means that the inside of the cells are slightly negative in relation to the outside. The resting potential is caused by the difference in ion concentration between the inside and outside of the cells (the K^+ -concentration is high inside the cells and low outside, while Na^+ has an opposite distribution), and the leakage of ions through selective ion channels, through which leakage of K^+ is the most important. When K^+ is leaking out of the cell, a negative electric force will arise inside the cell.

The cell membrane in the nerve cell contains voltage gated Na^+ -channels. They will open when the membrane depolarizes – meaning that Na^+ will flow into the neuron along the inward electrochemical gradient (higher Na^+ concentration on the outside and a negative membrane potential). The following influx of Na^+ overwhelms the dominant potassium efflux of the resting membrane. The influx of sodium leads to a positive feedback cycle – the opening of Na^+ -channels leads to Na^+ influx, depolarizing more of the cell membrane, opening more of the Na^+ -channels. In this way the depolarization will run along the nerve cell.

When the cell membrane is depolarized to $+55$ mV, the sodium influx will end because the inward force from the concentration difference and the outward electric force equalize each other. The Na^+ -channels close, voltage gated K^+ -channels open and the membrane repolarizes.

Voltage gated Ca^{2+} -channels are located close to the active zone of the nerve terminals. They open when the membrane is depolarized. The depolarization is normally caused by action potentials, but can be caused by pathological conditions, e.g. shortage of energy (the cell will not be able to maintain the difference in ion concentration across the membrane) or severe electrolyte disturbance. One can trigger the calcium influx in the terminals experimentally by depolarizing the cells, either by an electrode (Galvani did it with a charged scalpel) or

chemically by e.g. increasing the extracellular potassium concentration.

Calcium influx through the open Ca^{2+} -channels is driven by a very large inward electrochemical force. The influx will cause a marked rise in the local calcium concentration (more than thousand folds) in the active zone within a few hundred microseconds. The calcium entry triggers an interplay with a variety of proteins (e.g. the SNARE proteins) leading to vesicular release of transmitters.

Neurotransmitters

A neurotransmitter is a substance which has an effect on postsynaptic cells when it is released from the nerve terminals. The transmitters could be excitatory, meaning that they depolarize the target cell and possibly lead to a new action potential or inhibitory, in which case they hyperpolarize the target cell, making it harder to trigger action potentials. Glutamate and γ -aminobutyric acid (GABA) are the most common excitatory and inhibitory transmitters in the brain (Ottersen and Storm-Mathisen, 1984).

Excitatory transmitters – glutamate

Glutamate was first suspected to have an important transmitter role in the late 1950. Electrophysiological studies showed excitatory actions in neurons when glutamate was added (Curtis et al. 1960). Glutamate was not generally accepted as an excitatory transmitter before the 1980s. Contributing to establishing glutamate as a transmitter was that glutamatergic pathways were characterized with immunocytochemistry, and it was demonstrated that glutamate was released from nerve terminals by way of Ca^{2+} dependent exocytosis (Storm-Mathisen et al., 1983; Fonnum 1984; Storm-Mathisen et al., 1986; Ottersen et al., 1990).

Glutamate receptors

There are two categories of glutamate receptors. The ionotropic receptors, which directly open ion channels and the metabotropic receptors, which have indirect effects through second messengers. Glutamate always give excitatory postsynaptic responses through activation of the ionotropic receptors, while activation of metabotropic receptors have a modulatory effect on synaptic transmission; it can either increase or decrease synaptic activity, depending on the type of metabotropic receptor that is activated.

The major types of ionotropic glutamate receptors are named after the synthetic substances that activate them; alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA). The non-NMDA receptors gates cation channels that are permeable to sodium and potassium, and is responsible for the large early part of the excitatory postsynaptic potential (EPSP) – the fast depolarization of the postsynaptic membrane (Hollmann and Heinemann, 1994).

The NMDA receptors differ from the non-NMDA receptors in their characteristics. They are highly permeable to calcium in addition to sodium and potassium. Calcium has effects beyond

depolarizing the membrane. It activates calcium dependent enzymes and second messenger systems that can have a variety of effects in the cell.

Opening of a NMDA-activated channel usually requires that the membrane is depolarized before activation, because a voltage sensitive block caused by a magnesium ion must be removed. This means that NMDA receptors often are dependent on concomitant activation of other excitatory receptors to function. NMDA receptors also require a co-agonist, glycine or D-serine, in addition to glutamate for activation. NMDA receptor activation may lead to long-term changes in the neuronal function, but if they are strongly activated also to toxic effects.

Excitotoxicity

The excitatory synapse is a fine-tuned system. High extracellular concentrations of glutamate can lead to cell death, through “overactivation” of especially the NMDA receptors. This will lead to increased influx of Ca^{2+} , which will trigger “death cascades” in the neuron, e.g. mitochondrial dysfunction, which can generate free radicals, and activation of proteases that initiate apoptosis – cell death. Glutamate in toxic amounts can be seen in conditions like ischemic stroke and severe epilepsy. Therefore, there has been a goal to develop drugs which can protect the neuron from glutamate, such as glutamate receptor antagonists. Unfortunately most of these attempts have failed because of the severe side effects caused by blocking glutamate receptors: sedation, impaired memory, psychomotor retardation, cognitive impairment and even schizophrenia-like symptoms like hallucinations and psychosis (Doble, 1999). In other words, the glutamate effect should be neither too weak nor too strong.

The neurotoxic effect of glutamate is called excitotoxicity (Olney, 1969). It has been proved that the effect is due to activation of glutamate receptors, because adding specific antagonists for instance to a cell culture will prevent the cell death caused by glutamate (Choi et al., 1987).

The recirculation of glutamate

Because glutamate can potentially damage nerve cells, it is essential to efficiently remove it from the synaptic cleft where it activates the receptors (Danbolt, 2001). In addition, it is economical for the nerve cell if the released glutamate is transported back to the neuron for transmitter purposes. Also cellular glutamate uptake is necessary for keeping a high signal to noise ratio in synaptic transmission. Glutamate is mostly taken up across cell membrane by specialized transporter proteins. They are highly effective as they maintain a concentration gradient across the cell membrane of several thousand folds. Five such high affinity sodium dependent excitatory amino acid transporters (EAATs) have been cloned (Danbolt, 2001): EAAT1 (GLAST), EAAT2 (GLT), EAAT3 (EAAC), EAAT4 and EAAT5. EAAT1-3 are distributed almost throughout the whole brain, EAAT4 is confined to the cerebellum and EAAT5 is located only in the retina. EAAT1 and 2 are primarily located on astrocytes, while EAAT3 and 4 are mainly situated in somatodendritic compartments of neurons.

It is three possible pathways of removal of glutamate from the synaptic cleft. Firstly, it could be taken up by EAAT1 and EAAT2 in astrocytic processes surrounding the synapse. Secondly,

it could be taken up by glutamate transporters in the presynaptic membrane. Even though this glutamate transporter protein is not definitely identified, it has been shown that there is a transporter in the EAAT-family present in the presynaptic membrane as demonstrated by uptake of D-aspartate (a glutamate analogue) in the terminals (Gundersen et al., 1993; 1995) as well as by immunocytochemistry of EAAT2 (Chen et al., 2004; Suchak et al., 2003; Furnes et al., 2008). Thirdly, glutamate could be removed by glutamate transporters on postsynaptic dendrites, as observed for EAAT3 in several brain regions (Furuta et al., 1997; Shashidharan et al., 1997) and for EAAT4 in the cerebellum (Dehnes et al., 1998).

When glutamate is taken up by transporters in the astrocytic membrane, it starts a journey that may lead back to an excitatory vesicle in a nerve terminal. Inside the astrocyte, glutamate is transformed to glutamine by the glial specific enzyme glutamine synthetase (Norenberg MD and Martinez-Hernandez A, 1979; Derouiche A and Frotscher M., 1991)

Glutamine is then released from the astrocyte into the extracellular space through a system N glutamine transporter (SN) (Chaudhry et al., 2002). Glutamine has no effect on any receptors, and may be released with no risk of excitotoxicity. The released glutamine is then taken up into nerve terminals by a system A neuronal glutamine transporter (SAT) (Chaudhry et al., 2002). Inside the terminal it is converted back to glutamate by phosphate-activated glutaminase (PAG) which is located in the mitochondrial outer membrane (Kvamme et al., 2008). Finally glutamate enters excitatory vesicles through the vesicular glutamate transporters (VGLUTs) (Freneau et al., 2004). The glutamate is now recycled and again ready to be released into the synaptic cleft (for evidence for the glutamate glutamine cycle, see e.g. Pow and Crook, 1996).

Background of the project

Aspartate

At the time I started my PhD studies there were dissensions concerning whether aspartate was a neurotransmitter. Aspartate was known to have excitatory properties and there were several findings that indicated a transmitter role for aspartate (see Gundersen and Storm-Mathisen, 2000 for a thorough review).

Aspartate should fulfill the following four criteria to be called a neurotransmitter: Aspartate should (1) be synthesized in the neuron, (2) be present in the nerve terminal and released from synaptic vesicles by regulated exocytosis, (3) have a receptor effect which can be imitated by adding exogenous substance into the system, and (4) be removed from the extracellular space by specific mechanisms (Schwartz, 2000).

(1) Aspartate was known to be synthesized in the neuron from glutamate through oxaloacetate by aspartate aminotransferase (Kugler P, 1987; Martinez-Rodriguez and Arenas, 1988; Schmidbaur et al., 1990). In this reaction an amino group from glutamate is transferred to oxaloacetate, giving aspartate and α -ketoglutarate. When glucose is scarce oxaloacetate will accumulate. This explains why the aspartate/glutamate ratio was found to be increased when the energy consumption was larger than the supply, such as during high neuronal activity (Szerb JC, 1988) and hypoglycemia (Gundersen et al., 2001) (fig 2).

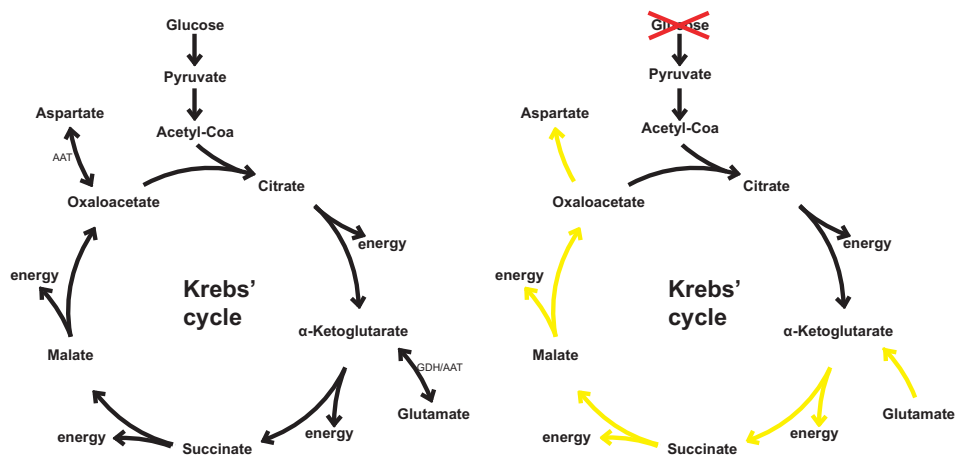


Fig 2: The flux of aspartate and glutamate through the Krebs' cycle in normal (left) and hypoglycemic(right) situations.

This means that glutamate formed through the glutaminase reaction should be a precursor of aspartate, but direct evidence for this was lacking. Moreover, whether glutaminase played a main role for the intraterminal production of glutamate and whether this applied to all excitatory terminals was not known. Opposite the the situation for glutamate-glutamine shuttle between neurons and astrocytes, evidence for a similar GABA-glutamine shuttle involving the glutaminase reaction in inhibitory terminals was scarce, as it was thought that a large part of GABA was derived from uptake across the plasma membrane through the GABA transporters (Bak et al., 2006).

(2) Concerning localization in excitatory nerve terminals electron microscopic studies showed inconsistent results. Even though several studies showed that aspartate was present in excitatory nerve terminals (Merighi et al., 1991; Tracey et al., 1991; Van den Pol, 1991; Usami and Ottersen, 1996, Gundersen, et al 1998, 2001), other studies found no evidence of such presence (Maxwell et al., 1990; Zhang et al., 1990; Ji et al., 1991; Montero, 1994; Larsson et al., 2001). It may be various reasons for this; different antibodies, different preparation procedures, varying concentration of aspartate in different neural pathways and the possibility that aspartate is not a transmitter in some of the terminals studied. The concentration of free aspartate in nervous tissue is significantly lower than for glutamate (about 1/5), and balances on the limit of detection with electron microscopic immunocytochemical techniques. Hypoglycemia in the brain increases the amount of aspartate, and varying glucose concentration in the animals could have been a reason for the different findings (Gundersen, 2001).

The question of whether aspartate had access to synaptic vesicles was unresolved. Immunogold cytochemistry had given evidence that aspartate was located in excitatory synaptic vesicles (Gundersen et al., 1998). However, most studies showed that aspartate was not taken up into preparations of isolated synaptic vesicles (for review, see Fykse and Fonnum, 1996). In addition, the vesicular glutamate transporters did not seem to transport aspartate (Freneau et al., 2004). Thus, further investigations of the uptake of aspartate in synaptic vesicles would throw important light on the transmitter status of aspartate.

Despite the negative results from the synaptic vesicle uptake studies, several investigations had showed that aspartate was released from depolarized nerve endings in a manner consistent with exocytosis (e.g. Gundersen et al., 1991, 1998), but some found no evidence for such a release (Nicholls, 1989, 1993). Thus, there was no general agreement about how aspartate was released (Nicholls and Attwell, 1990).

In particular, the observation of an exocytotic release of aspartate could be explained by the possibility that aspartate is released from the cytosol through the EAATs in exchange for glutamate released from synaptic vesicles. Thus, any manipulation that affects the release of glutamate would also affect the release of aspartate.

Although the strongest evidence of nerve terminal aspartate localization was from excitatory terminals, some studies indicated the presence of aspartate also in inhibitory GABAergic terminals (Ottersen and Storm-Mathisen, 1985; Gundersen et al., 1991, 2001). Whether aspartate was released from this type of terminal and by which mechanism was unknown.

(3) Aspartate was known to have a selective effect on the NMDA receptor, without activating any of the other glutamate receptor types (Curras and Dingledine, 1992). This means that aspartate could partly have a different role than glutamate when released into the synaptic cleft. Increased levels of aspartate, will lead to a shift towards a stronger NMDA-receptor stimulation. There was evidence that aspartate was released in increased amounts in hypoglycemia (Sandberg et al., 1986; Gundersen et al. 2001) and during increased neuronal activity (Szerb, 1988). This could mean that aspartate was responsible for parts of the excitotoxic effect seen in pathological conditions.

(4) Like for glutamate, if aspartate was a transmitter, it would be essential to keep the extracellular levels low in order to achieve an efficient signal transmission and to avoid excitotoxicity. This job was known to be done by the EAATs, all of which transport aspartate, both the D- and the L-form, with high affinities (Danbolt, 2001).

Low affinity aspartate/glutamate transport

Some studies had described a low affinity uptake system for aspartate and glutamate (see Danbolt, 2001). However, a more detailed characterization of the low affinity system was lacking. In particular, the identity of the transporters underlying the low affinity uptake was unknown. Also there was no information about which cell type in the brain that harbored low affinity uptake sites.

QUESTIONS TO BE ANSWERED

1. Is aspartate a transmitter at excitatory synapses?

Release through the EAATs?

Uptake of D-aspartate in synaptic vesicles? Synthesis, release and uptake of transmitter amino acids at central nervous synapses
2. Is aspartate a transmitter at inhibitory GABAergic synapses?

Location in synaptic vesicles?

Exocytotic release?

Presence of postsynaptic NMDA receptors?
3. Is glutaminase important for formation of transmitter L-glutamate, L-aspartate and GABA in nerve terminals?
4. Are there other uptake mechanisms than the EAATs for removing excitatory amino acids from the extracellular space?

METHODS

Brain slices

By incubating fresh slices of rat brain in a solution equivalent to extracellular fluid in the brain (Krebs' solution), it is possible to study the effect of different exogenous interactions (transporter substrates, membrane depolarisation, transmitter precursors etc). The Krebs' solution has approximately the same ion composition, glucose concentration, pH and osmolality as the cerebrospinal fluid.

The brain was harvested from a decapitated rat, quickly cooled in ice cold Krebs' solution and the part of interest, e.g. the hippocampus or cerebellum, dissected out and cut on ice with a tissue chopper in 0.3 mm thick slices, before putting the slices in the incubator (fig. 3).

During the incubation, the slices were submerged in continuously oxygenated Krebs' solution at 30° C on nylon grids in a beaker (fig. 4). Before starting any manipulation of the slices, they were pre-incubated in normal Krebs' solution for one hour. This pre-incubation is thought to let the cells recover after the preparation procedure.

An elegant way to study transporter proteins in the cell membranes is to add a substrate of the transporter and then reveal the intracellular localization of the added substrate. To recognize the exogenous substances from the endogenous substances radioactive substances could be used, or as I did, use substrates which do not naturally occur in the brain, and then detect the exogenous substance with immunocytochemistry (see below).

The use of brain slices has some limitations of which the problem with diffusion of molecules into the slice is important. The diffusion distance of a molecule is dependent on the size of molecule, the time allowed for diffusion (the distance is proportional to the square root of the time), the tortuosity of the tissue or whether the molecule can diffuse through lipid membranes. The size of the molecule is the factor which requires most attention. Small molecules, like glutamine and D-aspartate easily diffuse through the whole slice. Large molecules, like proteins diffuse only a very short distance into the slice and their effect can therefore only be studied on the slice surface.

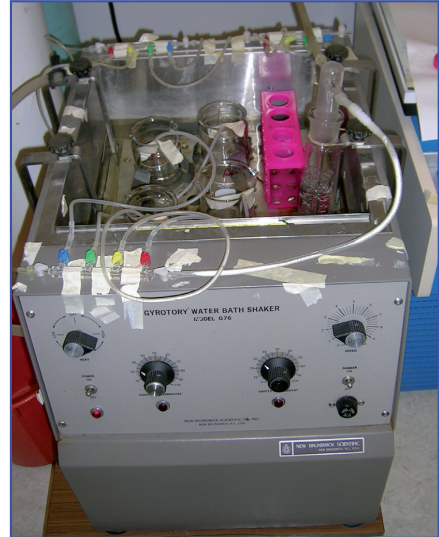


Fig 3: The set up for slice incubation

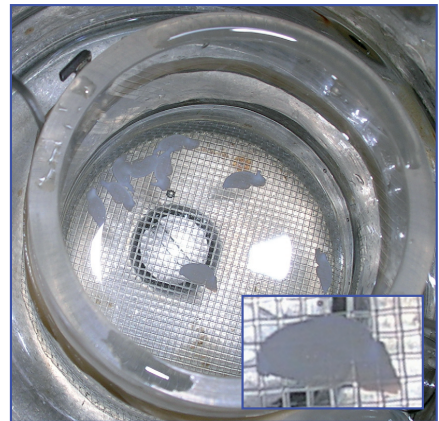


Fig 4: Hippocampal slices submerged in solution on a nylon grid. The inset shows the slice at higher magnification.

Embedding the tissue for electron microscopy

In order to study the tissue in the electron microscope very thin (about 100 nm thickness) tissue sections must be cut (ultrathin sections). This can be achieved from tissue that has been embedded in plastic resin. In this project I have embedded the tissue in Lowicryl HM20 at low temperatures (for detailed protocol, see Materials and Methods in Paper IV). The advantage of this embedding method is that it gives a good preservation of antigenic sites in the tissue, which in turn gives a high immunocytochemical labelling intensity. Some of the material was embedded in Durcupan. This procedure is less demanding to perform than the Lowicryl embedding, as it does not require a freeze-substitution unit. However, Durcupan embedded tissue shows lower immunocytochemical sensitivity, partly because the polymerisation step is done at high temperatures (56 °C). Ultrathin sections were cut on an ultramicrotome, mounted on nickel grids (300-500 mesh) and processed with the different antibodies according to an immunogold method (see below).

Perparing the tissue for light microscopy

After immersion fixing the hippocampal slices, they were cryo-protected in sucrose (30%) and 20 µm thick sections were cut on a freezing microtome. Then the sections were subjected to immunocytochemistry according to a three layer immunoperoxidase method (see below).

Immunocytochemistry

Immunocytochemistry with antibodies selectively recognizing amino acids is of great value when studying amino acid neurotransmitters. This method was originally described by Jon Storm-Mathisen in *Nature* in 1983 (First visualization of glutamate and GABA in neurons by immunocytochemistry). In this work glutamate and GABA were conjugated to bovin serum albumin (BSA) by glutaraldehyde and the antisera were raised by injecting these amino acid-glutaraldehyde-BSA conjugates in rabbits. By use of these antisera it was for the first time possible to immunocytochemically localize the transmitter amino acids in the brain. Since then this method has been the “state of art” method for morphologic identification of amino acid transmitters in the brain. It should be noted that the antibodies used in this thesis have been raised using a mixture of glutar- and formaldehyde to conjugate the amino acids to BSA. The resulting polyclonal antibodies react to different epitopes of these conjugates. Thus, a prerequisite for optimal antibody labelling is to use the same glutar- and formaldehyde mixture as a tissue fixative.

The first localization studies of amino acids were mostly done with the light microscopic immunoperoxidase method (Storm-Mathisen et al., 1983, Ottersen and Storm-Mathisen, 1984, 1985, Gundersen et al., 1991). This method is mostly used in a qualitative manner to decide if a tissue compartment contains the amino acids or not. The method could also give an impression of the relative concentration of the amino acid based on the intensity of the labelling, but this is most useful when comparing structures with large differences in amino acid concentrations (e.g. glutamate or aspartate in nerve terminals before and after a depolarization (Paper I and III)).

Most amino acid transmitters, like glutamate and aspartate, are besides being transmitters, building blocks of proteins and important in metabolic routes. These amino acids are present in all cells. Therefore, it could be difficult to separate the transmitter pool of amino acids from the metabolic pool, and small changes in the transmitter pool after synaptic release could thus be masked. However, the releasable pool of transmitter amino acids in the nerve terminal is packed in synaptic vesicles, in which it is located with a much higher concentration than in the metabolic pool of amino acids in the cytosol. Thus, the transmitter and metabolic pool of amino acids can be recognized by quantitative methods, such as the electron microscopic postembedding immunogold method. GABA has on the contrary a much more limited metabolic role. It acts to a large extent exclusively as a transmitter. The GABA-labelling is therefore not as widespread as the other amino acid transmitters, being localized mostly to inhibitory fibers in the brain. In many cases we are therefore more dependent on quantitative studies for localizing transmitter glutamate than transmitter GABA.

Accurate quantitative studies can be performed with a postembedding electron microscopic immunogold procedure (Bergersen et al, 2008), as this method, contrary to preembedding methods, is practically independent on penetration of the antibodies into the tissue section. Another great advantage of using this method is that it gives a high lateral resolution, which is required for studying labeling in subcellular compartments. The lateral resolution of the immunogold method (i.e. the distance between the epitope and the centre of the immunogold particle - approximately 20-30 nm) is much higher than the preembedding electron microscopic peroxidase method, in which the distance between the epitope and the peroxidase precipitate can be several micrometers.

Using the immunogold method I have compared the concentration of the substance of interest in different tissue compartments or in the same compartment in slices that have been treated differently. This can be done by counting the number of gold particles in the actual tissue compartment, determine the area of the compartment (by using computer programs or stereological methods) and then calculate the density of the gold particles (number of gold particles/ μm^2). To make a comparison of gold particle densities for an antigen between different compartments the density should be proportional to the concentration of the antigen fixed in the tissue (see below).

Specificity testing of antibodies against amino acids in light and electron microscopic immunocytochemistry

Antibodies are capricious, and they require a great level of caution when used in immunocytochemical procedures. Cross-reactivities and unspecific labeling can occur, and it is necessary with careful specificity testing. In quantitative studies it is also necessary to test that the antibody labeling of the amino acid is approximately proportional to the amino acid concentration in the tissue.

If one works with tissue labeling of proteins, the most thorough and ultimate specificity test of the antibodies is to use tissue from an animal in which the protein in question is knocked out. Another possibility is to test the antibodies on cultured cells, which either express or do not express the particular protein (Bergersen et al., 2001). None of these tests are usually possible on amino acid antibodies. Most amino acids have several synthetic pathways, which cannot

be knocked out. An important exception is when working with labeling of exogenous amino acids that accumulate in tissues not endogenously containing the amino acid, like D-aspartate in the hippocampus (see below; Paper IV).

Antibody testing on tissues lacking the antigen

D-aspartate has a very low endogenous concentration in the forebrain, including the hippocampus (Gundersen et al., 1993), and is transported by the excitatory amino acids transporters in the same manner as L-glutamate and L-aspartate (Danbolt, 2001). I studied the glutamate/aspartate uptake in the brain by adding exogenous D-aspartate to the slices. Since D-aspartate is slowly metabolized in the forebrain (Davies and Johnston, 1975) it is “trapped” within the compartment in which it was taken up. Thus, it is possible to detect the localization of D-aspartate by using immunocytochemistry with antibodies recognizing the amino acid. Such a D-aspartate uptake assay is therefore well suited for studying uptake of excitatory amino acids in the brain (Gundersen et al., 1993, 1996).

When I labelled sections from hippocampal slices not incubated in D-aspartate with the 402 D-aspartate antiserum there was no visible

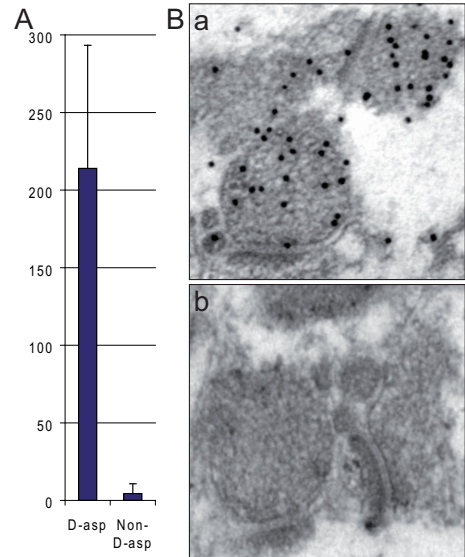


Fig 6: A. The bars indicate the difference in gold particle density (average number of gold particles/μm² ±SD) between sections from slices incubated in D-aspartate and those from slices not incubated in D-aspartate. B. Electron micrographs show D-aspartate immunogold labeling of two nerve terminals from a slice incubated in D-aspartate (a) and a slice not incubated in D-aspartate (b) (from work performed in Paper IV).

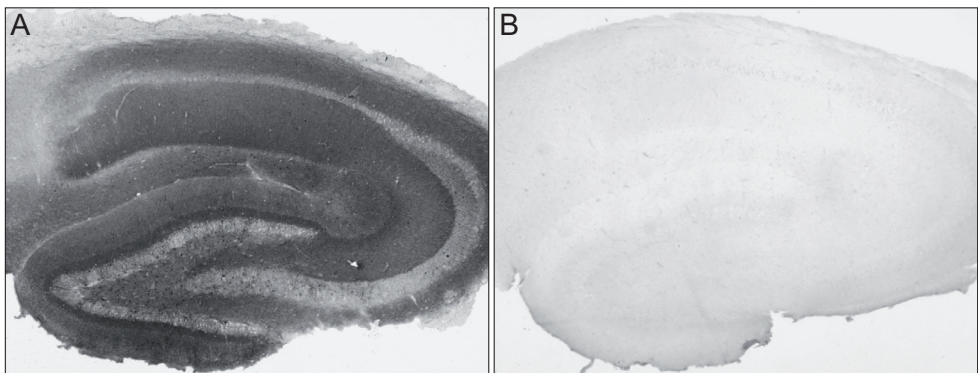


Fig 5: Sections from the work described in Paper IV. Section in A is from a hippocampal slice incubated in 0.5 mM D-aspartate in 15 min. Section in B underwent the same treatment as A, except that D-aspartate was not added to the incubation medium. Both are labeled in the same experiment according to the immunoperoxidase protocol with the 402 antiserum against D-aspartate. Note the lack of specific labeling of the section in B (only the shadows of the hippocampal layers and some edge staining of the section can be seen).

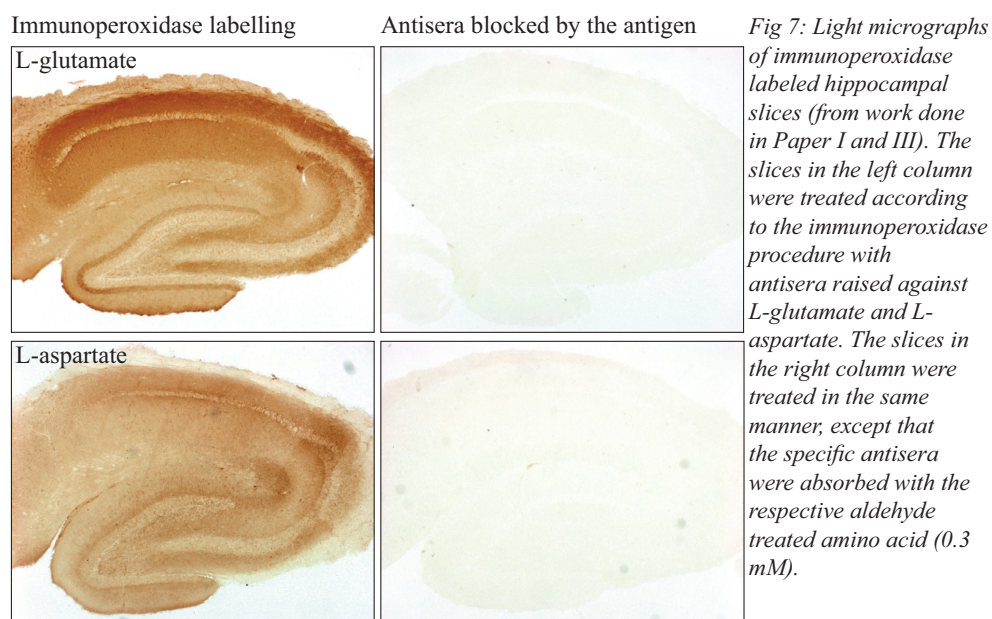
D-aspartate labelling in the light microscope, whereas the D-aspartate treated slices showed significant staining (fig 5). In the electron microscope there was immunogold labelling particular in nerve terminals and astrocytes in the D-aspartate treated hippocampus, whereas the labelling in the non-D-aspartate exposed tissue was at background level (fig 6). This indicates that the D-aspartate antiserum does not cross react with any molecule present in the forebrain, which also was confirmed by specificity “spot testing” (see below). Thus, I conclude that the D-aspartate antiserum is highly specific.

Absorbing the specific antibodies

A commonly used specificity test is to absorb the antibodies with the antigen prior to applying the antibodies in the immunocytochemical procedure. I made soluble glutar-and formaldehyde complexes of the amino acids in question, which were added to the antibody solution before performing the immunocytochemical procedure. This treatment blocked the specific binding site of the antibody. Thus, any remaining labeling should be due to unspecific binding. I used this test both in the light and electron microscopic immunocytochemical procedures.

However, this test has a potential weak point. Our experience is that the binding site of one antibody could recognize more than one amino acid; the one that it were originally raised against, but also other amino acids, which may get a similar antigen structure when conjugated with aldehydes to albumin. This “incorrect” labeling will also disappear when the binding site is blocked. Therefore, this method could not be entirely trusted if used alone. The test is a good complement to the “spot test” (see below).

I have used this “absorption method” in all my immunohistochemistry work. In fig 7 the method is applied for the antibodies against L-glutamate and L-aspartate.



Cross reactivity tests

In most cases, working with amino acid antibodies, the most important test are those involving conjugates resembling the antigens.

The light microscopic “spot test”

In my test system I fixed L-glutamate, L-aspartate, glutamine and D-aspartate to brain proteins by formaldehyde and glutaraldehyde, forming amino acid – protein conjugates. The amino acid conjugates were spotted on a small piece of cellulose nitrate-acetate filter paper by a micro pipette. This spot test was performed along with each set of immunoperoxidase experiments. For practical reasons only the four most relevant antigens were tested for each antiserum. One spot, containing fixed brain macromolecules without any amino acids, were also added (named “none”). However, all our antisera have previously been tested against more than 40 small molecules known to be abundantly present in the brain (Ottersen, 1986).

While working with Paper I, II and III I tested the following antisera: 607 L-glutamate (dilution 1:5000), 435 L-aspartate (dilution 1:2000) and 990 GABA (dilution 1:1000) (these antisera have also been previously characterized in Gundersen et al., 1998, 2001). The 402 D-aspartate antiserum (dilution 1:500) was tested while working with Paper IV (these antibodies have been extensively tested in Gundersen et al. (1993)). All antibodies tested (607 L-glutamate, 435 L-aspartate, 990 GABA and 482 D-aspartate) were highly specific. All spot tests showed only one labeled spot (the one that contained the amino acid against which the antiserum was raised), except the spots stained by the L-aspartate antiserum, which showed a slight cross reaction with D-aspartate. However, this does not result in any cross-labelling in the tissue sections since the brain regions studied in this thesis contain only trace amounts of D-aspartate (Hashimoto et al., 1995). In fig 8 results of staining the spots with the L-aspartate, L-glutamate and D-aspartate is given as examples of the spot tests.

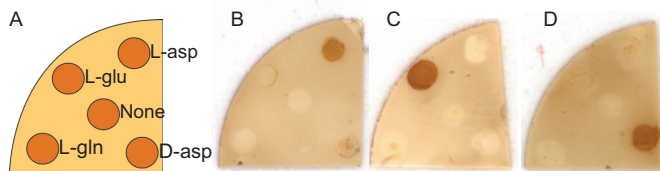


Fig 8: “Spot test”. A, Figure showing the antigens of each “spot”. The quarto pieces of paper where labelled with antisera against L-aspartate (B), L-glutamate (C) and D-aspartate (D).

Electron microscopic specificity test

The electron microscopic specificity test is in principle equal to the “spot test”. The antibodies are tested against different structurally related amino acids, which occur in the brain at high levels (see Ottersen, 1987).

In our electron microscopic test system, the amino acid-glutaraldehyde/formaldehyde-brain protein conjugates (containing L-aspartate, L-glutamate, GABA, glutamine, taurine, glycine and fixed brain proteins without any amino acid (“none”)) were mounted in layers with sections from rat brain between (Ottersen, 1989b; fig 9). The amino acid concentration in the conjugates is about 100 mM (Ottersen, 1987). This “sandwich” was embedded in Durcupan, and ultrathin sections were cut on an ultratome. The ultrathin sections were subjected to the postembedding immunogold method along with the ultrathin tissue sections described in Paper I, II and III. In Paper IV the 402 D-aspartate antiserum was tested in the same way, but

the “sandwich” contained a different battery of amino acid conjugates (see Gundersen et al., 1993, 1995, 1996).

Results of labeling with the L-aspartate antiserum are given as an example of specificity testing using the electron microscopic test system (fig 9, from work conducted in Paper III). The GABA and L-glutamate antisera have the similar specificity (Paper I, II and III). The density of immunogold particles over the L-aspartate conjugate produced by the L-aspartate antiserum (dilution 1:50) was very much higher than the densities over the other amino acid conjugates. From this I conclude that the L-aspartate antiserum is specific when tested in the electron microscopic test system (cf. the spot test above).

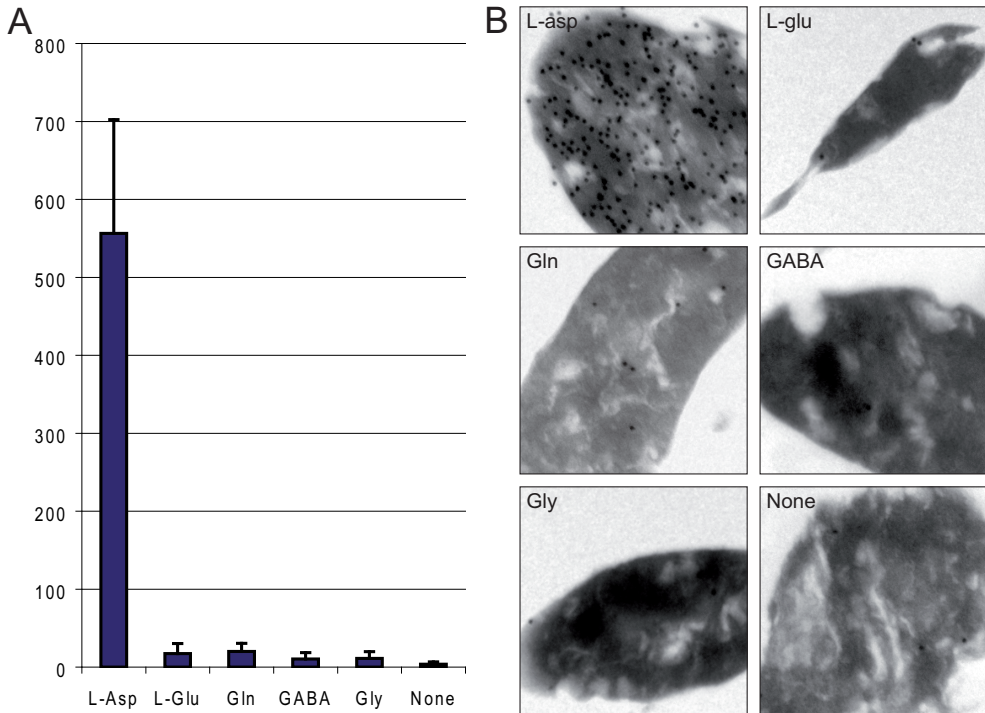


Fig 9: A: Immunogold labeling with the antiserum against L-aspartate. The bars show the gold particle density (average number of gold particles/ $\mu\text{m}^2 \pm \text{SD}$) over conjugates of amino acids fixed to brain macromolecules. B: Electron micrographs of the immunogold labeled conjugates.

Estimation of the relationship between gold particle density and antigen concentration

The conjugates could also be used to test the relationship between the gold particle density and the concentration of the fixed amino acid. In this test the conjugates do not contain different amino acids, but different concentration of the same amino acid. All our antibodies tested till now have shown a close to linear relationship between the density of immunogold particles over the test conjugates and the amino acid concentration fixed in the conjugates (see Ottersen, 1989a). Below I describe the linearity test of the L-aspartate antiserum. Such a test was performed in Paper I, II and III for the 435 L-aspartate antiserum (see Gundersen et al.,

1998, 2001 for previous tests). Similar tests have also been performed with the 990 GABA antiserum (Paper II) and previously with the 607 L-glutamate antiserum (Ericson et al., 1995; Gundersen et al., 1998) and 402 D-aspartate antiserum (Gundersen et al., 1995).

Conjugates where the L-aspartate concentrations have been estimated to be about 0, 0.04, 0.1, 0.3, 1.0, 3.0, 9.0 and 27.0 mM were put together in layers with sections of brain tissue as spacers between. The stack was embedded in Durcupan, ultrathin sections were cut and processed with the 435 L-aspartate antiserum according to the immunogold method.

The results for the L-aspartate antibodies are very similar to those previously obtained for glutamate antibodies (Ottersen, 1989a; Bramham, 1990). The slope is almost linear at antigen concentrations between 3-30 mM (fig 10). Nerve terminals in the brain contain L-aspartate within this concentration range (Paper II; Gundersen et al., 1998). Thus, when studying the L-aspartate content in nerve terminals by immunocytochemistry (Paper I, II, III) the immunosignal is positively correlated with the concentration of the amino acid.

In Paper I and II I used such graphs, in combination with the immunogold particle density in tissue compartments, to estimate the amino acid concentration fixed in the tissue. However, some precautions must be taken when doing this. Mostly I used tissue which has been embedded in Lowicryl, whereas the graded test sections were embedded in Durcupan (Ottersen, 1989b). Thus, the tissue sections mostly give a stronger immunogold signal than the test sections. Second, a certain proportion (about 30-50 %) of the amino acids are lost during the fixation procedure (Storm-Mathisen and Ottersen, 1990), meaning that one must do an approximation in order to estimate the concentration of the free amino acid in the tissue.

This test could also be used to get a rough estimation of the sensitivity of the labeling; i.e. which amino acid concentration that is necessary to get a signal significantly higher than background. The gold particle density (the number of L-aspartate immunogold particles/ μm^2) in the example in fig 10 was higher than the background (0 mM L-aspartate) at 0.3 mM L-aspartate. The average tissue concentration of L-aspartate is about 2 mM (the L-glutamate concentration is 5 times higher) (Nadler et al., 1978), whereas the concentration in nerve terminals is higher (the concentration of fixed L-aspartate in nerve terminals is about 3-4 mM (Paper II; Gundersen et al., 1998), giving a concentration of about 6-8 mM of free L-aspartate in the terminals). The sensitivity of the L-aspartate antibodies is in other words sufficient for transmitter studies.

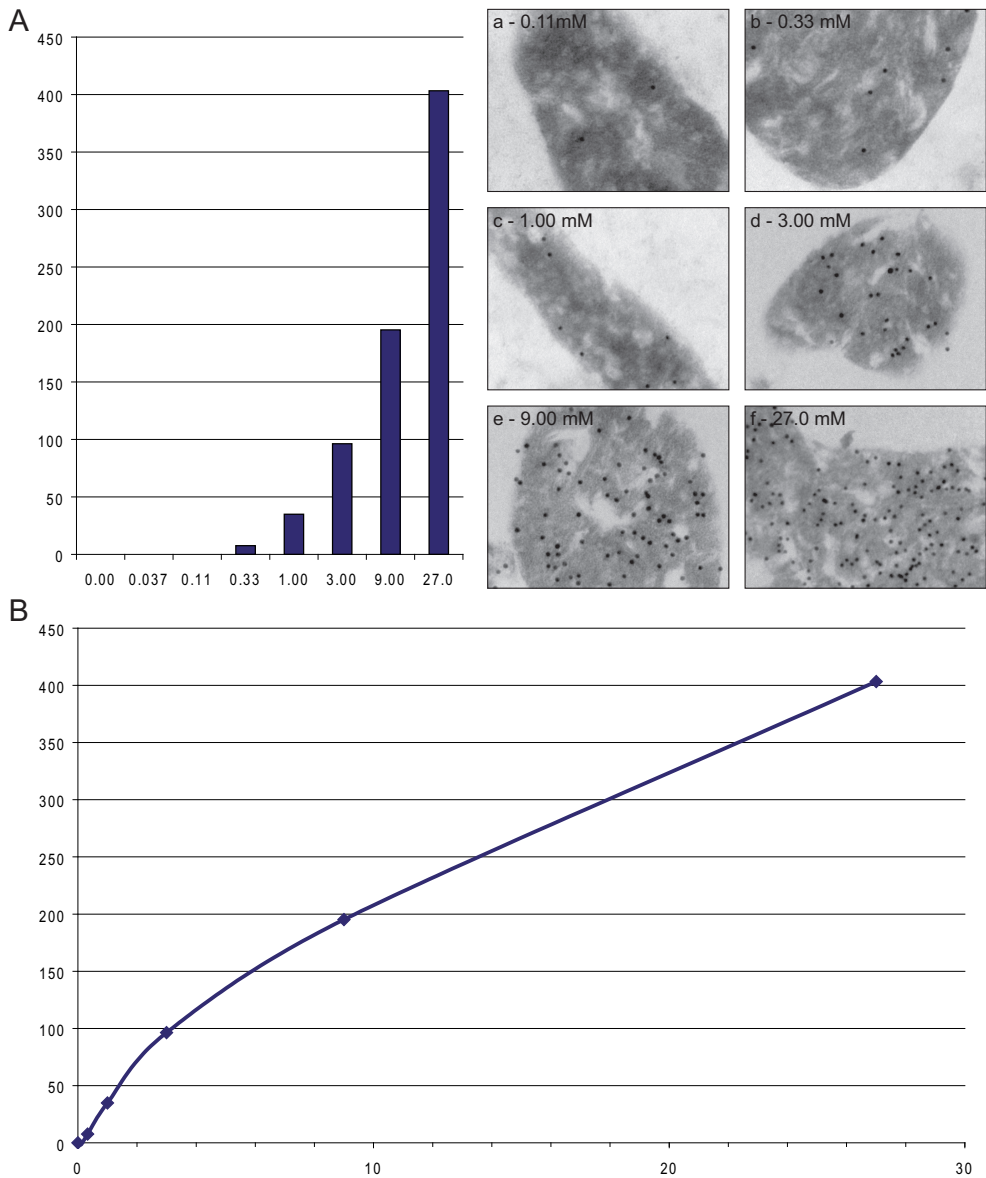


Fig 10: A. Gold particle densities signaling L-aspartate (number of gold particles/ μm^2) over conjugates with different L-aspartate concentrations. a-f are electron micrographs of the graded L-aspartate conjugates showing that the density of the gold particle labeling increases with increased amino acid concentration in the conjugates. B shows the relationship between the fixed antigen (L-aspartate) and the gold particle densities. The detection limit of the L-aspartate immuogold method is about 0.3 mM.

SUMMARY OF RESULTS

Paper I

There are convincing data substantiating that L-aspartate is released by exocytosis from excitatory nerve terminals. The release, which is triggered by depolarization, is dependent on calcium and the SNARE proteins. However, it has been argued that the same findings could be seen if L-aspartate was released by heteroexchange against exocytotically released glutamate through the excitatory amino acid transporters (EAATs). To test this theory we blocked the EAATs in depolarized hippocampal slices. The release of L-aspartate from excitatory terminals was only weakly affected by this blocking, further adding to the evidence of aspartate exocytosis from these terminals.

A major argument against exocytotic release of aspartate was until recently that no vesicular aspartate transporter was described. The vesicular glutamate transporters (vGLUTs) do neither transport L- nor D-aspartate. In the present paper we showed that exogenously D-aspartate added to hippocampal slices is taken up and stored inside vesicles. There is no known route in the hippocampal neurons which can metabolize and synthesize D-aspartate, meaning that the exogenous added D-aspartate must have been transported into the vesicles by an aspartate transporter. This vesicular transporter carries probably both D- and L-aspartate. Our results fit with the recent finding of a vesicular aspartate transporter in hippocampal synaptic vesicles.

Paper II

There are strong evidence that aspartate could act as a transmitter at excitatory synapses. Previous immunocytochemical studies link aspartate also to GABAergic neurons. In the present study we used the immunogold method to detect aspartate and GABA in hippocampal slices. We could show that under resting conditions the excitatory transmitter aspartate was present in synaptic vesicles in GABAergic terminals, and that aspartate and GABA were depleted from the same terminals during potassium induced depolarization. In the absence of calcium or in the presence of tetanus toxin (both preventing exocytosis) the depletion of both GABA and aspartate was blocked.

Since the NMDA-receptor is the only receptor activated by aspartate, we investigated if this receptor was located in the GABAergic synapses. By immunogold double labeling, we showed that NMDA receptors were present in GABAergic synapses. We propose that exocytotically released aspartate could play a physiological role in the GABAergic, inhibitory synapses.

Paper III

An important part of the recirculation of glutamate between nerve terminals and astrocytes is thought to be played by the enzyme phosphate activated glutaminase, which turns glutamine into glutamate in the terminals.

In this paper we inactivated glutaminase in cerebellar slices with the use of DON. By this we showed a significant reduction in glutamate, aspartate and GABA in terminals during membrane depolarization in spite of the presence of glutamine, which is a substrate of glutaminase. This means that the synthesis of glutamate, aspartate and GABA is dependent on the glutaminase reaction.

Paper IV

By incubating hippocampal slices with millimolar concentrations the glutamate analogue D-aspartate and pharmacologically blocking the high affinity glutamate transporters (excitatory amino acid transporters – EAATs) we showed that low affinity glutamate transporters are present in astrocytes in the hippocampus. D-aspartate uptake activity was detected by D-aspartate immunocytochemistry. When incubating hippocampal slices in a low concentration of D-aspartate (0.01 mM), D-aspartate was taken up into nerve terminals and astrocytes. When adding PMB-TBOA, a potent EAAT blocker, no sign of D-aspartate was evident. When the slices were incubated with PMB-TBOA during increased D-aspartate concentrations (0.5 and 1.0 mM) the nerve terminal uptake was blocked, but a significant uptake remained in the astrocytes. We conclude that this PMB-TBOA insensitive astrocyte uptake of D-aspartate represents a low affinity uptake, which is only active when the levels of extracellular glutamate and aspartate are raised to millimolar concentrations.

Further characterization of the PMB-TBOA insensitive uptake showed that removing the sodium ions in the incubation solution entirely blocked the uptake. This indicates that the transporter responsible for the PMB-TBOA insensitive uptake in astrocytes is sodium dependent. When adding succinate, in addition to PMB-TBOA, to the D-aspartate exposed slices the low affinity transport was significantly reduced. These characteristics fit with the idea that a Na^+ /dicarboxylate cotransporter is located in the astrocyte membrane and contributing to the observed low affinity aspartate/glutamate uptake.

DISCUSSION

Release of aspartate from nerve terminals

Aspartate is present in nerve terminals and is released during depolarization (see Introduction). However, there has been dissension about the actual release mechanisms. The confirmation of this is a crucial point for determining its transmitter status. There are at two possible release mechanisms:

- A) Through exocytosis
- B) Through a channel or a transport protein

The following discussion will mainly be based on the role of aspartate at excitatory synapses. However, the similar arguments hold true for the role of aspartate at inhibitory synapses (see below).

A) There is plenty of evidence indicating that release of aspartate through exocytosis is important. First, immunocytochemistry of hippocampal slices has shown that aspartate is localized in and released from synaptic vesicles in excitatory (Gundersen et al., 1998; Yatsushiro et al., 1997) and inhibitory terminals (Paper II). In addition, there are convincing biochemical data showing Ca^{2+} -dependent release of aspartate in brain slices and in the intact brain (see Gundersen and Storm-Mathisen, 2000). In contrast, there have been some conflicting data concerning the Ca^{2+} -dependency of aspartate release in synaptosomes (see Nicholls and Attwell, 1990). However, evidence have accumulated during the last years that aspartate is released by exocytosis also from synaptosomes (e.g. McMahon et al., 1992; Bradford and Nadler, 2004; Wang and Nadler, 2007). Importantly, aspartate release is inhibited by clostridium toxins (McMahon et al., 1992; Gundersen et al., 1991; Wang and Nadler, 2007), of which tetanus toxin degrades synaptobrevin and botulinum toxin cleaves several proteins of the SNARE complex (Schiavo et al, 1992). These proteins are necessary for the vesicle fusion with the cell membrane.

There has for a long time been one important missing link in the theory of exocytotic release of aspartate. How does aspartate get into the vesicles? Several studies have tried to demonstrate a vesicular aspartate uptake, most of them by measuring inhibition of vesicular glutamate uptake. Aspartate did not inhibit the glutamate uptake into isolated synaptic vesicles from the whole brain (Naito and Ueda, 1983, 1985; Maycox et al., 1988; Tabb and Ueda, 1991; Fykse et al., 1992; Burger et al., 1989). Thus, the conclusion was drawn that aspartate is not taken up in vesicles. However, one study reported that aspartate is transported into synaptic vesicles (Fleck et al., 2001a). The fact that the vesicular glutamate transporters (vGLUTs) exclusively seem to transport glutamate (Freneau et al., 2004), as well as that the vesicle uptake of aspartate and glutamate is not inhibited by the other, and that their uptake affinities are somewhat different (Fleck et al., 2001a), supports the possibility that aspartate is taken up into synaptic vesicles by another transporter than the vGLUTs. Recently, this assumption proved to be correct. Miyaji et al. (2008) demonstrated that a H^+ /sialic acid co-transporter pumps aspartate into synaptic vesicles. They have renamed it vesicular excitatory amino acid transporter (VEAT). In addition to sialic acid, VEAT transports L-aspartate as well as L-glutamate, and D,L-threo-hydroxy aspartate (THA) (see Fleck et al., 2001b). The localization of VEAT in the intact brain is not yet established, but the authors have shown that

the transporter is present in synaptic vesicles isolated from the hippocampus as well as from pinealocytes (the latter cells store and release both aspartate and glutamate (Yatsushiro et al., 1997)).

As VEAT transports the D-aspartate analogue THA (Miyaji et al., 2008) it may be envisaged that it also transports D-aspartate. This would fit nicely with the D-aspartate immunogold data presented in Paper I and with data showing D-aspartate uptake in isolated synaptic vesicles (Fleck et al., 2001a), as well as with the fact that D-aspartate can be released by exocytosis after it has been preloaded into different brain tissue preparations (Malthe-Sørenssen et al., 1979; Drejer et al., 1983; Potashner, 1983; Minc-Golomb et al., 1987; Cousin et al., 1997; Cousin and Nicholls, 1997; Rousseau et al., 2005; Raiteri et al., 2007). Furthermore, THA reduces the release of L-aspartate, probably by an effect on the vesicular aspartate uptake system, but has no effect on the L-glutamate release (Fleck et al., 2001a).

One study (Bradford and Nadler, 2004) has shown that aspartate release, in contrast to glutamate release, is not blocked by bafilomycin. Bafilomycin is a bacterial toxin (from *Streptomyces griseus*) which specifically inhibits the vesicular H^+ -ATPase. Glutamate transport through the vGLUTs is driven by the electrical component set up by the H^+ electrochemical gradient over the vesicular membrane. The vesicular H^+ -ATPase creates this driving force. However, the results of Bradford and Nadler (2004) do not fit with the properties of VEAT, which like the vGLUTs, is driven by the electrochemical gradient across the vesicle membrane ((Miyaji et al., 2008). It should be noted that the data in Bradford and Nadler (2004) indicated that the release of aspartate was much less sensitive to clostridium toxins than that of glutamate. However, in a later publication Victor Nadler shows that after electroporation tetanus toxin does indeed inhibit the release of aspartate (Wang and Nadler, 2007), suggesting that the former results were due to limited access of tetanus toxin (and perhaps of bafilomycin) to the nerve terminals.

B) There is an alternative hypothesis of aspartate release from nerve endings; release through a protein transporter. Because of the strong evidence of a Ca^{2+} -dependent and clostridium toxin sensitive release, a simple one-way transport (e.g. through reversal of EAATs) responsible for the total release of aspartate is unlikely. However, aspartate release could be inhibited by means that block exocytosis if it occurs secondary to synaptic glutamate release. When glutamate is released, the extracellular concentration of glutamate would temporarily be very high. This could trigger a heteroexchange with cytosolic aspartate through the EAATs located in the nerve terminal membranes (Gundersen et al., 1993; Furness et al., 2008; Paper IV).

There are several pieces of data that weaken the heteroexchange theory. First, immunogold cytochemistry show that aspartate is concentrated in synaptic vesicles rather than in the cytosol (Paper II, Gundersen et al., 1998). In Paper I we have blocked the EAATs with the potent blocker PMB-TBOA. If aspartate was dependent on the EAATs for release (through heteroexchange) the release should be terminated by this blocker. However, we observed that significant amounts of aspartate were released in nerve terminals during membrane depolarization, even if the EAATs were blocked. In Paper I we discovered that 30-36% of aspartate and glutamate released from the excitatory terminals could be blocked by blocking the EAATs. This probably represents release through reversal of the EAATs (during the high K^+ -conditions) and fits well with earlier findings, which have shown that the residual release of aspartate and glutamate during blocking of exocytosis makes up about 30-40 % of the

exocytotic release (Gundersen et al., 1998).

There are, in my opinion, two possible mechanisms for vesicular aspartate accumulation. Glutamate could be transformed to aspartate inside the vesicles. However, this is probably the least probable explanation. Firstly, aspartate aminotransferase is found in mitochondria (McKenna et al., 2000), but there is no evidence of any vesicular localization. Secondly, as discussed above exogenous D-aspartate accumulates in synaptic vesicles (Paper I). Even though the possibility exists that L-aspartate could be created from L-glutamate in the vesicles, conversion of L-aspartate to D-aspartate is highly unlikely, as it would require an intrasynaptic racemase. The activity of D-aspartate racemase is very low in the adult forebrain (Davies and Johnston, 1975).

I therefore consider the first possible mechanism for vesicular aspartate accumulation for the most probable; namely through a vesicular aspartate transporter. Indeed, the recent identification of VEAT (Miyaji et al., 2008) greatly supports this notion, but more work has to be done to settle this (particularly thorough localization studies to see if all proposed aspartergic terminals in the brain contain VEAT).

Thus, taking an excitatory synapse as an example (fig 11), glutamate is formed from glutamine through phosphate activated glutaminase (see below). Then aspartate is produced from glutamate via aspartate aminotransferase (AAT). The vGLUTs exchange glutamate against protons, while aspartate is transported by another vesicular transporter, probably VEAT. The aspartate uptake is also dependent on H^+ -ATPase if VEAT is the dominant vesicular aspartate transporter. In fig 11 the two transporters are indicated in the same vesicle for simplicity.

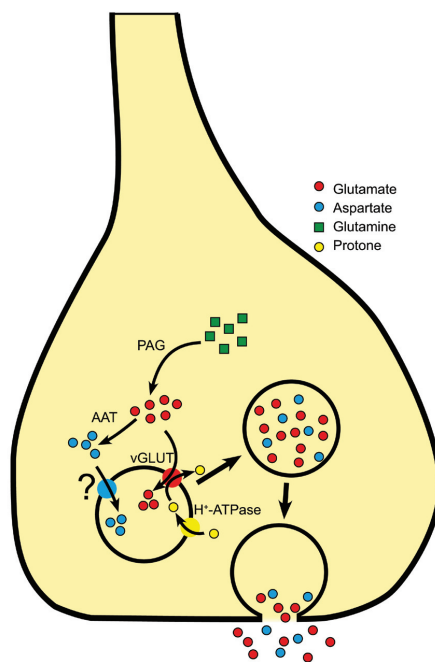


Fig 11: Cartoon showing the mechanisms for exocytotic release of glutamate and aspartate.

The synthesis of glutamate, aspartate and GABA

Laake et al. (1999) showed low levels of phosphate activated glutaminase (PAG) in the glutamatergic parallel fibers in the cerebellum. They observed that the parallel fiber terminals contained similar PAG levels as GABAergic terminals. These levels were only about 20 % of the PAG level seen in the cerebellar mossy fibers. The low PAG levels in the glutamatergic parallel fiber terminals (and the GABA terminals) correspond poorly with the theory of recirculation of glutamate (and GABA) between nerve terminals and astrocytes, where PAG is an important step.

However, in Paper III we found that glutaminase is necessary to produce releasable glutamate

from glutamine during synaptic activity. This seems like a contradiction to the findings of Laake et al. (1999), who used antibodies against the kidney type of PAG (see below), and the immunogold method to detect and quantify the levels of the enzyme. However, they determined the relative amount of the enzyme, not the glutaminase activity. In Paper III we studied the activity of the enzyme by observing the increased level of the product (glutamate) when the substrate (glutamine) was added and then the absence of the product when the enzyme was blocked.

There could be several reasons why the parallel fibers have lower levels of the kidney PAG than the mossy fiber terminals. As there is not necessarily any strict relation between the amount of enzyme and its activity it could be that the low density of PAG in the parallel fiber terminals has an activity high enough to sustain the glutamate levels during synaptic release.

Moreover, it is possible that the parallel fiber terminals contain isoenzymes of glutaminase that were not recognized by the antibodies used by Laake et al. (1999). There are two established isoenzymes, the kidney and the liver type (Aledo et al., 2000). Laake et al (1999) used antibodies against the former type.

It should also be mentioned that the parallel fiber terminals are tightly ensheathed by Bergmann glial processes (Grosche et al., 2002), which contain EAAT1 (Lehre et al., 1995) that actively take up glutamate during synaptic transmission (Clark and Barbour, 1997; Takayasu et al., 2004). Interestingly, the glial processes around parallel fibers contain much higher densities of EAAT1 than the processes around mossy fiber terminals (Chaudhry et al., 1995). In addition, EAAT4 is located in Purkinje cell dendrites postsynaptic to the parallel fiber terminals (Dehnes et al., 1998), suggesting that most of the synaptically released glutamate at the parallel fiber synapse is taken up by other EAATs than those present on nerve terminals. This suggests that a minor fraction of the synaptically released glutamate enters the terminal by reuptake, and that the parallel fiber terminals are dependent on replenishment of transmitter glutamate via the glutamate-glutamine cycle and a glutaminase reaction in the terminals. This is in line with our finding that the parallel fiber terminals contain high glutaminase activities. In contrast, the astrocytic ensheathment of the mossy fiber synapse is less developed than that of the parallel fiber synapse (Peters et al., 1991). However, despite that little is known about glutamate transporters on nerve terminal plasma membranes in the cerebellum, it seems as if the mossy fiber terminals contain less uptake sites for glutamate than the parallel fiber terminals (Wiklund et al., 1982). In other words, even though there probably is more available glutamate around mossy than parallel fiber terminals, poor uptake capacity prevents the mossy fiber terminals to depend on a reuptake. This may explain why also mossy fiber terminals possess high levels of the glutaminase enzyme (Laake et al., 1999; Paper III).

In Paper III we also showed that the release of aspartate from glutamatergic terminals in the molecular (parallel fiber terminals) and granule (mossy fiber terminals) layer of the cerebellum and that the release of GABA from inhibitory terminals is dependent on PAG. These results further support the notions that aspartate can be a transmitter at central synapses (Paper I, II) and that glutamine is an important precursor for releasable GABA. When working with Paper III we noticed PAG-dependent aspartate localized in inhibitory Golgi cell terminals in the granular cell layer. Such a presence of aspartate in inhibitory GABAergic terminals is in line with data presented in Paper II (also see Gundersen et al., 2001).

Aspartate in inhibitory synapses

I have in this thesis argued for aspartate as an excitatory transmitter. It is substantial evidence that aspartate is present in and released from excitatory nerve terminals, probably by exocytosis. Paper II shows that aspartate is localized in and released by exocytosis from inhibitory nerve terminals along with GABA. The exocytotic co-release of aspartate and GABA, as well as of aspartate and glutamate, is in line with evidence that other fast acting neurotransmitters are co-released from synaptic vesicles: glycine and GABA (Jonas et al., 1998), ATP and GABA (Jo and Schlichter, 1999) and glutamate and acetylcholine (Nishimaru et al., 2005) in spinal cord synapses; glutamate and GABA in mossy fiber synapses (Walker et al., 2001; Bergersen et al., 2003). Recently, Boulland et al. (2008) demonstrated that a vesicular glutamate transporter and the vesicular GABA transporter were both present in the same nerve terminal in the dentate gyrus of the hippocampus.

There could, however, be alternative explanations of the exocytosis dependent depletion of aspartate from GABAergic synapses. Firstly, aspartate could be released by reversed transport through amino acid plasma membrane transporters during depolarization. However, this explanation is less likely as excitatory amino acid transporters (EAATs) seem not to be present in the membranes of GABAergic terminals (Gundersen et al., 1993). In addition, our observation that the aspartate release from GABA terminals is Ca^{2+} -dependent and tetanus toxin sensitive speaks against release by reversed transport. However, we cannot strictly rule out the possibility that aspartate could be removed from the GABA-terminals in an indirect manner: Aspartate could be converted to glutamate by the enzyme aspartate aminotransferase. Glutamate is a precursor for GABA via a reaction catalyzed by glutamate decarboxylase. Therefore, it is possible that aspartate is consumed to meet the demand of releasable GABA. However, it seems like aspartate is mainly localized in vesicles, and is thus inaccessible for aspartate amino transeferase.

Aspartate has an effect exclusively on the NMDA type of glutamate receptors (Curras and Dingledine, 1992). This means that aspartate released at a glutamatergic synapse will lead to a relatively stronger activation of NMDA receptors compared to that of AMPA receptors. In the GABAergic synapses aspartate will be responsible alone for the NMDA receptor activation, which could serve as a tool for fine regulation of the extent of excitatory and inhibitory input to a neuron. In this respect it should be noted that NMDA receptor activation could occur even if the Mg^{2+} block is not removed by concomitant depolarization of the synaptic membrane (Parri et al., 2001; Karadottir et al., 2005; Jourdain et al., 2007). Aspartergic NMDA receptor activation could be important for e.g. synaptic potentiation phenomena, such as long term potentiation.

Uptake of excitatory amino acids from the extracellular space

In Paper IV we demonstrated that D-aspartate was transported into astrocytes by an EAAT-independent low affinity uptake system. The fact that the uptake was sodium dependent and reduced by succinate, suggests that a Na^+ /dicarboxylate cotransporter, which transports aspartate and glutamate with low affinities (for review, see Markovich and Murer, 2004), is responsible for the observed D-aspartate uptake. Thus, in the following I call this D-aspartate uptake mediated by the dicarboxylate transporter “aspartate/glutamate uptake” (cf. the

substrate specificities for the EAATs (Danbolt et al., 2001)). As NaC3 has been shown to be present in cultured astrocytes (Fujita et al., 2005), I think that this transporter underlies the transport of aspartate/glutamate in our experiments. In the course of the work with Paper IV, we tried to localize NaC3 by immunocytochemistry, using a commercial antibody, but we did not succeed. We suspect that the antibodies were not of high enough sensitivity.

As succinate could not entirely block the observed D-aspartate accumulation, I should emphasize that there could be additional low affinity aspartate/glutamate transporters mediating excitatory amino acid uptake in astrocytes. Because we found that homocysteate also to a certain extent could inhibit the low affinity aspartate/glutamate uptake such a transporter could belong to the ASC transporters (Budy et al., 2006).

Since the extracellular aspartate/glutamate concentration must be around 0.5 mM to give an considerable influx through NaC3 (Huang et al., 2000; Paper IV) a possible function of this low affinity transport system could be to help regulating the uptake of excitatory amino acids after synaptic release, during which the concentration of aspartate/glutamate can rise to mM concentrations (Danbolt, 2001). Also in pathological conditions, where the extracellular concentrations of aspartate/glutamate are high, this low affinity uptake system can be important (see below).

It has to be emphasized that the main function of NaC3 and the other dicarboxylate transporters is probably not uptake of excitatory amino acids, but transport of Krebs' cycle intermediates (preferably dicarboxylates with four or five carbon chain length). Succinate is one of the high affinity substrates of this transporter. The chemical structure of aspartate differs only from succinate by aspartate's amino group. However, this one positively charged group gives a large reduction in affinity (Huang et al., 2000; Wang et al., 2000; Burckhardt et al., 2005; Fujita et al., 2005). When NaC3 expressed in mammalian cell lines were tested for inhibition succinate transport, millimolar concentrations of aspartate and glutamate could significantly inhibit succinate uptake by about 40 % and 20 %, respectively (Huang et al., 2000; Wang et al., 2000). Thus when the concentrations are high it seems like NaC3 transports the excitatory amino acids quite efficiently. This fits with the extensive low affinity aspartate/glutamate uptake observed in astrocytes in Paper IV.

The low affinity uptake system seem to transport aspartate more efficiently than glutamate. Aspartate reduces succinate (Huang et al., 2000; Wang et al., 2000) and N-acetyl-L-aspartate (another high affinity NAC3 substrate) (Fujita et al., 2005) uptake through NaC3 more efficiently than glutamate. Thus, NaC3 has a lower K_m for aspartate than glutamate. This could be of importance during certain pathological conditions, since aspartate selectively activates the NMDA receptors, and could therefore at high concentrations give a relatively stronger contribution to excitotoxicity than glutamate.

Pathological conditions

The above described aspartate exocytosis from excitatory and inhibitory nerve terminals and the low affinity uptake system for excitatory amino acids could be involved in various pathological states in the brain.

Aspartate seems to selectively activate NMDA receptors (Curras and Dingledine, 1992). In addition, the ratio of aspartate release to glutamate release increases during high neuronal stimulation (Szerb, 1988; Szerb and O'Regan, 1987) and when brain energy levels are low, such as during hypoglycemia (Gundersen et al., 2001). Thus, during these conditions the high release of aspartate may shift the activation of glutamate receptors towards higher NMDA receptor activation. An "overactivation" of NMDA receptors is detrimental to the brain and involved in the development of several disease states.

Epilepsy

E.g. during status epilepticus it is shown that inhibition of hippocampal granule cells is lost. This loss can be annulled by NMDA receptor antagonists (Mazarati and Wasterlain, 1997). Thus, it could be that during epilepsy an increased NMDA receptor activation of granule cells, which can be mediated by an enhanced release of aspartate, could neutralize or even reverse the inhibitory GABA effect. Therefore, this could make inhibitory synapses excitatory and participate in the uncontrolled excitatory activity during epilepsy. Further adding to this notion is that it is known that mice genetically predetermined for epilepsy, but not yet experienced seizures, have a high release of aspartate (Flavin and Seyfried, 1994).

Brain ischemia

High extracellular excitatory amino acid concentrations cause massive stimulation of the glutamate receptors, particularly the NMDA receptors. This could lead to degeneration of neurons because of excessive calcium influx through the activated receptors. This is called excitotoxicity (Olney, 1969), which is one of the main factors underlying the death of neurons during e.g. brain ischemia (Mattson, 2003).

The brain contains huge amounts of putative toxic excitatory amino acids, which are trapped inside intracellular vesicular compartments by energy dependent processes. When the energy balance in the brain is broken down during ischemia, these excitatory amino acids represent a risk if they are released into the extracellular space. Also if the extracellular concentrations of aspartate/glutamate are allowed to build up during normal synaptic transmission, they may represent a potential danger to the neurons. Thus, the brain balances between excitatory transmission and excitotoxicity. However, the brain has evolved protective systems, e.g. represented by the EAATs, against the harmful effects of aspartate and glutamate. This can be demonstrated by adding exogenous glutamate receptor agonists to the brain. For example, adding of kainate, which is a poor substrate of the EAATs, to the striatum will lead to extensive neuronal death (Coyle and Schwarcz, 1976; Obrenovitch et al., 2000). However, simple adding of glutamate, which will be taken up by the EAATs, requires much higher concentrations to create cell death, and the affected region will be much smaller. In a study by Mangano and Schwarz (1983) high glutamate concentrations (in the molar range) were added to the rat striatum repeatedly every 12 hour in 14 days, but only a minor degeneration was seen. In contrast, in pure neuronal cultures, where astrocytes containing efficient protective glutamate uptake systems are lacking, a much smaller concentration of glutamate will kill the neurons (50-100 μ M (Choi et al., 1987)). If the neuronal EAATs are blocked in such cultures, then glutamate concentrations in the lower μ M range are sufficient to cause neuronal death (Frandsen and Schousboe, 1990). Moreover, in conditions where the uptake of excitatory amino acids is compromised, such as during lack of energy, excitatory amino acids are severely toxic to the neurons (Novelli et al., 1988).

During physiological conditions, in which extracellular glutamate and aspartate mainly comes from synaptic release, the EAATs comprise the most important protective system for removing the amino acids from the extracellular space. During pathological conditions, in which there is a massive efflux of excitatory amino acids (a situation mimicked by Mangano and Schwarz (1983)) the low affinity uptake system for excitatory amino acids (Paper IV) will probably also be important. The low affinity system has K_m values of about 1 mM (Logan et al., 1972; for NaC3 kinetics, see Huang et al., 2000), whereas the K_m values of the high affinity system (EAATs) are in lower micromolar range (Danbolt, 2001). The high affinity system would probably not be able to increase its transport activity when the extracellular glutamate approaches millimolar concentration, because it is saturated at much lower concentrations. Low affinity uptake is far from saturated, and can take a greater part in the removal of extracellular glutamate during conditions with elevated glutamate concentrations.

During brain ischemia a deficient Na^+/K^+ -ATPase-function could lead to depolarization of cells of the brain. Depolarization and increased intracellular Ca^{2+} concentration lead to a massive exocytosis of aspartate/glutamate and eventually to reversal of excitatory amino acid transporters. Glutamate in the astrocytes will not be transformed to glutamine, because glutamine synthetase requires ATP. Thus, glutamate will accumulate in the astrocytes, which in turn will lead to an increased driving force for reversal of glial EAATs, or at least make the inward transport less efficient. In turn this will lead to further increases in the extracellular concentrations of aspartate and glutamate. In Paper IV we detected that the low affinity system transports excitatory amino acids at concentration down to 50 μM . As it also efficiently transports aspartate/glutamate at millimolar concentrations, this system could therefore contribute to the prevention of excitotoxicity during brain ischemia.

Such a role will require that the low affinity transporters, including the Na^+ /dicarboxylate transporters, can take up excitatory amino acids during ischemic conditions. Net uptake through the high affinity excitatory amino acid uptake system is less efficient when the ion gradients across the plasma membrane are reduced. At least the high affinity transport can reverse during ischemia and massive depolarization (Rossi et al., 2000; Allen et al., 2004). The transport through NaC3 is, like the EAATs, driven by an inward sodium co-transport (three Na^+ ions per dicarboxylate molecule). During membrane depolarization this uptake decreases because of reduced transmembrane potential difference (tested for succinate uptake, not for aspartate or glutamate)(Wang et al., 2000). However, in intact tissue it seems like the astrocytes can accumulate aspartate/glutamate through the low affinity Na^+ /dicarboxylate transport system, even if their membranes are depolarized by high K^+ concentrations (Paper IV). The low affinity transporters could mediate this inward transport of aspartate/glutamate through a Na^+ dependent net uptake, or if these transporters, like the EAATs convey heteroexchange (see Furness et al., 2008), through a Na^+ independent heteroexchange with intracellular succinate.

CONCLUSIONS

1. Aspartate is a transmitter at excitatory synapses:

The release of L-aspartate from excitatory nerve terminals is mainly by way of exocytosis, only a minor fraction escapes through reversal of the EAATs.

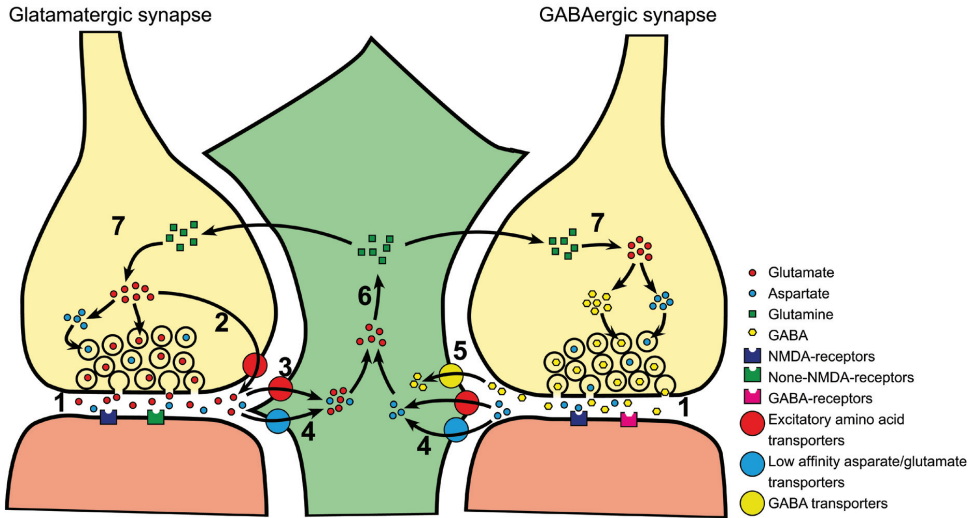
D-aspartate is transported into excitatory synaptic vesicles

2. Aspartate is a transmitter at GABAergic synapses:

Aspartate is present over synaptic vesicles and depleted by exocytosis.

NMDA-receptors are present in postsynaptic dendrites.

3. During synaptic transmitter release the nerve terminal content of glutamate, as well as that of aspartate and GABA, is dependent on synthesis from glutamine through the enzyme glutaminase.
4. During episodes of raised extracellular levels of aspartate and glutamate, the low affinity transport system transports the excitatory amino acid into astrocytes. Na^+ /dicarboxylate cotransporters are probably important transporters in this system. Thus, the EAATs are probably not the only plasma membrane transporters important for clearing aspartate and glutamate from the extracellular space.



Aspartate (blue) and glutamate (red) or aspartate (blue) and GABA (yellow) are released from glutamatergic and GABAergic nerve terminals (Paper I, II and III), respectively. The co-release of aspartate and glutamate are shown to occur by exocytosis (1), but release through the excitatory amino acid transporters (2) also contributes (Paper I). GABA and aspartate are released via exocytosis from GABAergic terminals (1) (Paper II). After release aspartate and glutamate are transported into surrounding astrocytes (Paper I, III) by excitatory amino acid transporters (3) and low affinity aspartate and glutamate transporters (4) (Paper IV), whereas GABA is transported by GABA transporters (5). In astrocytes glutamate is converted to glutamine by glutamine synthetase (6). Aspartate and GABA are first converted to glutamate, which in turn forms glutamine through the glutamine synthetase reaction. Then glutamine is transferred to nerve terminals by various glutamine transporters. In the terminals glutamine could act as a precursor of glutamate, aspartate and GABA through phosphate activated glutaminase, which are active in both glutamatergic and GABAergic terminals (7) (Paper III).

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